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Phylogenetic Relationships among Diverse Dinoflagellate Species Occurring in Coastal Waters off Korea Inferred from Large Subunit Ribosomal DNA Sequence Data

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We analyzed the nuclear-encoded large subunit ribosomal RNA gene (LSU rDNA) sequences of 19 dinoflagellates occurring in costal waters off Korea and reconstructed a phylogenetic tree containing 74 representative species from 37 distinct genera. Of these, the LSU rDNA sequences of *Amylax triacantha* (Jörgensen) Sournia, *Gonyaulax verior* Sournia (= *Amylax diacantha* Meunier), *Gyrodinium fissum* (Levander) Kofoid et Swezy, *Katodinium glaucum* (Lebour) Lebour III, *Noctiluca scintillans* (Macartney) Kofoid et Swezy, *Oxyphysis oxytoxoides* Kofoid, and *Pyrophacus steinii* (Schiller) Wall et Dale are reported for the first time. Our LSU rDNA tree consistently placed *Oxyrrhis marina* Dujardin and *N. scintillans* at the most primitive positions, giving rise to a strongly supported monophyletic group of typical dinoflagellate species belonging to the Dinophyceae. The phylogenetic relationships among the typical dinoflagellates, however, were not resolved in the higher taxonomic levels in general. Only genera at terminal branches were usually supported with high confidence. The Dinophysiales, represented by *Dinophysis* species and *O. oxytoxoides*, formed a strongly supported monophyletic assemblage. The Gymnodiniales and Peridiniales were recovered as polyphyletic groupings. Members of the Gonyaulacales were consistently grouped together, but lacked statistical support. Within this order, the Ceratiaceae and Goniodomataceae each formed a monophyletic group, but the Gonyaulacaceae was polyphyletic. The phylogenetic relationships of the Gonyaulacaceae were generally congruent with differences in the combinations of the apical pore complex, hypothecal organization and thecal formula.

Key Words: dinoflagellates, LSU rDNA, phylogeny

INTRODUCTION

Alveolata is a strongly supported monophyletic group unified by cortical alveoli and tubular mitochondrial cristae and comprises three well-defined clades: Apicomplexa, Ciliophora and Dinozoa (Cavalier-Smith 1993; Patterson 1999; Adl *et al.* 2005). The latter is further subdivided into three lineages: Dinoflagellata, *Oxyrrhis* Dujardin, and Perkinsidae (Adl *et al.* 2005). Of these, Dinoflagellata is characterized in the motile stage as possessing two dimorphic flagella, i.e., a 'whirling' transverse flagellum set into the cingulum and a posterior longitudinal flagellum set into the sulcus, a dinokaryotic nucleus lacking essential histones (dinokaryon), and condensed chromosomes during interphase (Fensome *et al.* 1993, 1999; Taylor 2004; Adl *et al.* 2005). They inhabit diverse aquatic ecosystems, have a variety of trophic behaviors such as autotrophy, heterotrophy, mixotrophy, myzocytosis, phagotrophy, parasitism, and symbiosis, and play important ecological roles in aquatic habitats as both primary producers and consumers. Many form harmful algal blooms (HABs) that devastate aquatic animals, resulting in tremendous economic losses to local fishery industries, or produce potent toxins that contaminate fish or shellfish and threaten human health (Hallegraeff 1993; Van Dolah 2000).

Phylogenetic studies based on molecular sequence data have provided great insights into the evolutionary history and systematics of divergent life forms by providing an objective taxonomic criterion. The nuclear-encoded large subunit ribosomal RNA gene (LSU rDNA) contains both conserved and divergent domains (Wuyts *et al.* 2001). This molecule has been used to address the ambiguous phylogenetic relationships of dinoflagellates at broad taxonomic levels (e.g., Scholin *et al.* 1994; Daugbjerg *et al.* 2000; de Salas *et al.* 2003; Ellegaard *et al.* 2003; Flø Jørgensen *et al.* 2004a, 2004b; Kim *et al.* 2005b; Gribble and Anderson 2006). The accumulation of LSU rDNA data enables the identification of dinoflagellates at

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the species level and the tracing of the dispersal of toxic marine microorganisms (e.g., Scholin *et al.* 1994; Medlin *et al.* 1998; Higman *et al.* 2001). Moreover, rDNA molecules are generally suitable for the molecular identification and quantification of target microorganisms because of the high number of copies per genome and huge database for comparative analysis. Many specific oligonucleotide probes or primer sets for rapid and accurate HAB monitoring have been designed based on rDNA sequence information (e.g., Rehnstam-Holm *et al.* 2002; John *et al*. 2003; Kim *et al*. 2004a). However, most sequence information is restricted to cultivable photosynthetic dinoflagellate taxa. Half of dinoflagellates are heterotrophic (Taylor 1987) and difficult to culture, resulting in their inevitable exclusion from molecular phylogenetic studies. To overcome this problem, several research groups have used a single-cell PCR technique to amplify target gene(s) from a single motile cell (Bolch 2001; Takano and Horiguchi 2005).

We incorporated new sequence information on the LSU rDNA D1-D3 region of 19 diverse dinoflagellate species that frequently occur in coastal waters off Korea. The sequence information was used in a currently available sequence matrix to elucidate the phylogenetic relationships. The rDNA molecule was PCR-amplified from extracted genomic DNA for cultured species, whereas PCR was begun from a single free-living cell or cyst germinant for heterotrophic or uncultivable species.

MATERIALS AND METHODS

Algal cell culture and single-cell isolation

A single motile dinoflagellate cell from a net-haul sample was removed using a micropipette under an inverted light microscope (Axiovert 200, Zeiss, Germany) and transferred to a test tube containing f/2-Si medium (Guillard and Ryther 1962). The algal culture was maintained at 20°C and ca. 50 μmol photons m^{-2} s^{-1} of cool white light under a 14L:10D photoperiod. At monthly intervals, a small portion of the culture was transferred to a test tube containing fresh medium.

For heterotrophic or uncultivable dinoflagellate species, a motile cell from a net-haul sample or a cyst germinant was removed using a micropipette, and identified to species and photographed under a light microscope (Olympus BX50, Olympus, Japan) with differential interference contrast (DIC) optics. Each cell was sequentially rinsed with sterile seawater and distilled water, and finally transferred to a PCR tube in the smallest possible volume for single-cell PCR (Bolch 2001). Two independent PCR runs and cycle-sequencings were carried out for dinoflagellate species applied to single-cell PCR to confirm their genetic identities. The two sequences for each dinoflagellate species were treated as single sequences when they were 100% identical. The dinoflagellate species investigated are listed in Table 1.

For consistent comparisons we applied the reinterpreted thecal formula of Kim *et al.* (2005a) for members of the Gonyaulacales. Briefly, according their reinterpretation, the Q platelet is treated as an auxiliary platelet, and the first postcingular plate (1”’) in the strict Kofoidean system (Kofoid 1911) is interpreted as a component of the sulcus, i.e., the accessory left sulcal platelet (Ssa). Finally, the posterior intercalary plate (1p) is included in the antapical plate series; thus, the 1p and the first antapical plate (1””) in the Kofoidean system are homologues of the 1”” and the second antapical plate (2””), respectively.

PCR and sequencing

The genomic DNA of a culture sample was extracted from cells in late-exponential phase following the methods of Hong *et al.* (1995), with slight modification, i.e., the addition of a phenol:chloroform:isoamyl alcohol (25:24:1) step. A PCR tube containing a single dinoflagellate cell was subjected to freeze-thaw cycles to rupture the cell wall prior to PCR according to Bolch (2001). The following PCR reagents were added to a PCR tube containing 1 μl of genomic DNA or a single cell in a 50-μl reaction volume: 1 × *Ex Taq*™ buffer, 200 μM dNTPs, 0.2 μM primers (D1R: 5’-ACCCGCTGAATTTAAGCATA-3’, Scholin *et al.* 1994; D3B: 5’-TCGGAAGGAACCAGCTACTA-3’, Nunn *et al.* 1996), and 1.25 U of *TaKaRa Ex Taq*™ (TaKaRa, Japan). PCR was initiated by a denaturation step at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 1 min; and completed with an elongation step at 72°C for 7 min. After gel purification of the appropriate PCR band using a QIAquick™ Gel Extraction kit (Qiagen, Germany), a cycle-sequencing reaction was performed using the PCR and internal primers, using an ABI PRISM BigDye™ Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Perkin Elmer, USA) according to the manufacturer’s instructions. Sequencing was conducted on an ABI 3100 Sequencer (Applied Biosystems).

Phylogenetic analysis

The LSU rDNA sequences were aligned with those of

Table 1. Information of dinoflagellate species investigated in this study

Species	Strain code	Sampling area	Sampling date	DNA source	GenBank acc. no.
Akashiwo sanguinea	GSW0207	Goseong	Jul 2002	culture	EF613348
Amylax triacantha	JHW0205-5	Jinhae Bay	May 2002	a motile cell	EF613350
Dinophysis acuminata	BSW0304	Busan	Apr 2003	a motile cell	EF613351
Gonyaulax verior	JHW0205-4	Jinhae Bay	May 2002	a motile cell	EF613349
Gymnodinium catenatum	JHW9910	Jinhae Bay	Oct 1999	culture	EF613352
Gyrodinium fissum	JHW0305-4	Jinhae Bay	May 2003	a motile cell	EF613353
Gyrodinium instriatum	JHW0007-2	Jinhae Bay	Jul 2000	culture	EF613354
Heterocapsa triquetra	GSW0206-2	Goseong	Jun 2006	culture	EF613355
Katodinium glaucum	JHW0305-1	Jinhae Bay	May 2003	a motile cell	EF613356
Lingulodinium polyedrum	DRW0108	Deungnyang Bay	Aug 2001	culture	EF613357
Noctiluca scintillans	GSW0205	Gunsan	May 2002	a motile cell	EF613358
Oxyphysis oxytoxoides	JHW0203-3	Jinhae Bay	Mar 2002	a motile cell	EF613359
Oxyrrhis marina	BSW0303	Busan	Mar 2003	a motile cell	EF613360
Prorocentrum micans	GSW0208	Goseong	Aug 2002	culture	EF613361
Protoceratium reticulatum	JHW0007-6	Jinhae Bay	Jul 2000	culture	EF613362
Pyrophacus steinii	JHW0007-3	Jinhae Bay	Jul 2000	culture	EF613363
Protoperidinium leonis	BAC0304c	Buan	Apr 2003	a cyst germinant	EF613364
Protoperidinium punctulatum	JHW0205-6	Jinhae Bay	May 2002	a motile cell	EF613365
Scrippsiella trochoidea	GSW9808	Goseong	Aug 1998	culture	EF613366

all dinoflagellate sequences available in GenBank (http://www.ncbi.nlm.nih.gov/) using BioEdit (Hall 1999) and edited manually with reference to the secondary structure information of *Toxoplasma gondii* Nicolle et Manceaux (Wuyts *et al.* 2001). A final alignment matrix was generated with representative dinoflagellate taxa. When the analyzed sequences of dinoflagellates overlapped those from GenBank, only the former sequence information was included in the phylogenetic analysis because of computational limitations. Finally, a matrix of 74 representative sequences from 37 distinct genera and 780 positions was assembled, excluding positions corresponding to the PCR primer regions and the ambiguously aligned D2 domain. The apicomplexan *T. gondii* (GenBank accession number X75453) was used as the outgroup.

A neighbor-joining (NJ) analysis was performed using PAUP* 4.0b10 (Swofford 2002), using the maximum-likelihood distance model. The Akaike Information Criterion (AIC) in Modeltest 3.07 (Posada and Crandall 1998) was used to determine the best fitting evolutionary model of nucleotide substitution. A NJ tree was constructed with the general time-reversible model allowing invariant sites and a gamma distribution (i.e., the GTR + I + Γ model), with the following likelihood settings: base frequencies of A = 0.2613, C = 0.1715, G = 0.2915, T = 0.2757; base substitution rates of AC = 1.5194, AG = 3.0930, AT = 1.2662, CG = 0.7974, CT = 8.7933, GT = 1.0000; assumed proportion of invariable sites = 0.1314; and gamma shape parameter = 0.8102. A bootstrap analysis was performed to statistically test the branching patterns with 1,000 pseudoreplicates (Felsenstein 1985).

The AIC model selection strategy implemented in MrModeltest 2.2 (Nylander 2004) was used to select the best fitting model for the Bayesian inference (BI) analysis. The BI analysis was conducted in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) using six rate classes of nucleotide change, invariable sites, and gamma distribution shape (corresponding to the GTR + I + Γ model). Two independent Markov chains were performed with the simultaneous four Metropolis-coupled Markov chain Monte Carlo (MCMC) chains (one cold and three heated), with random starting trees for 10,000,000 generations and sampling of trees at intervals of 100 generations. The first 1,000 of the 100,000 resulting trees were discarded as "burn-in" and the remaining trees were used to estimate posterior probabilities (PP).

RESULTS AND DISCUSSION

LSU rDNA sequence analysis

We determined the LSU rDNA sequences of 19 diverse dinoflagellate species that frequently occur in coastal waters off Korea (Table 1). Some were PCR-amplified starting from a motile cell in the water column or a cyst germinant. PCR amplification of the LSU rDNA D1-D3 region using the universal primer set consistently produced a single PCR band (data not shown). Two inde-

pendent PCR runs and cycle sequencings were carried out for each dinoflagellate species to confirm its genetic identity.

BLASTN searches in GenBank revealed that the LSU rDNA sequences of the following species showed the highest similarity (in parentheses) to their corresponding species, as expected: *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup GSW0207 (96.5%), *Dinophysis acuminata* Claparède et Lachmann BSW0304 (100%), *Gymnodinium catenatum* Graham JHW9910 (99.4%), *Gyrodinium instriatum* Freudenthal et Lee JHW0007-2 (99.5%), *Heterocapsa triquetra* (Ehrenberg) Stein GSW0206-2 (99.5%), *L. polyedrum* DRW0108 (89.4%), *Prorocentrum micans* Ehrenberg GSW0208 (100%), *Oxyrrhis marina* Dujardin BSW0303 (98.7%), *Protoceratium reticulatum* (Claparède et Lachmann) Bütschli JHW0007-6 (99.4%), *Protoperidinium leonis* (Pavillard) Balech BAC0304c (85.0%), *Protoperidinium punctulatum* (Paulsen) Balech JHW0205-6 (99.5%), and *Scrippsiella trochoidea* (Stein) Loeblich III GSW9808 (96.2%). The relatively high intraspecific sequence divergences in *L. polyedrum* and *P. leonis* were comparable to that among three *Ceratium* Schrank species (10.0-12.6%).

The LSU rDNA sequences of *Amylax triacantha* (Jörgensen) Sournia JHW0205-5, *Gonyaulax verior* Sournia JHW0205-4 (= *Amylax diacantha* Meunier), *Gyrodinium fissum* (Levander) Kofoid et Swezy JHW0305-4, *Katodinium glaucum* (Lebour) Lebour III JHW0305-1, *Noctiluca scintillans* (Macartney) Kofoid et Swezy GSW0205, *Oxyphysis oxytoxoides* Kofoid JHW0203-3, and *Pyrophacus steinii* (Schiller) Wall et Dale JHW0007-3 are presented here for the first time.

Overall phylogeny

Phylogenetic trees were reconstructed using the NJ and BI methods for the LSU rDNA sequence matrix, including the apicomplexan *T. gondii* as an outgroup, to elucidate the genetic relatedness among the dinoflagellate lineages (Fig. 1). The phylogenies inferred from the two algorithms had similar topologies (data not shown). The molecular phylogenetic tree revealed that all dinoflagellates formed a monophyletic group with respect to the apicomplexan outgroup. The dinoflagellates *O. marina* and *N. scintillans* were placed at the most primitive positions, giving rise to dinoflagellate species belonging to the Dinophyceae. The "typical" dinoflagellates formed a strongly supported monophyletic group with 99% bootstrap support (BS) in the NJ tree and 0.99 PP in the BI tree. Inter-relationships among typical dinoflagellates were fundamentally irresolvable, with no significant statistical support. Genera at terminal branches were supported with high confidence in the NJ (> 50% BS) and BI trees (> 0.95 PP).

Phylogeny of *Oxyrrhis marina* and *Noctiluca scintillans*

Oxyrrhis marina had an extremely divergent LSU rDNA sequence and, along with *N. scintillans*, was located at the most basal position relative to the typical dinoflagellates (Fig. 1). Although often considered a dinoflagellate because of its permanently condensed chromosome, *O. marina* possesses a number of atypical dinokaryotic features such as a pair of laterally inserted flagella, the lack of a true girdle and sulcus, intranuclear mitotic spindles, and the presence of histone proteins (Dodge and Crawford 1971; Triemer 1982; Fensome *et al.* 1993). Saldarriaga *et al.* (2003) and Leander and Keeling (2004) found that *O. marina* was paraphyletic to *Perkinsus marinus* at the most basal branches of typical dinoflagellates based on concatenated protein-encoding genes such as hsp90, actin, and β-tubulin. These results are consistent with the separate taxonomic placement of *O. marina* from the typical dinoflagellates (Fensome *et al.* 1993; Adl *et al.* 2005).

Noctiluca scintillans was one of the earliest diverging dinoflagellates in the LSU rDNA phylogeny, in contrast to its unstable position in a small subunit (SSU) rDNA phylogeny (Saldarriaga *et al.* 2004). *Noctiluca* Suriray has been speculated to be an ancestral dinoflagellate taxon because of its typical eukaryotic mitosis and chromosome, but is considered a dinoflagellate because of its gymnodinioid gametes with dinokaryotic dimorphic flagella and the presence of a nucleus during a part of the life cycle (Zingmark 1970; Pfiester and Anderson 1987; Höhfeld and Melkonian 1995). This atypical taxon was separated from typical dinoflagellates and placed in a different taxonomic group, the Noctilucales (Adl *et al.* 2005).

The pleomorphic life cycle of the endoparasitic *Amoebophrya* Köppen of the Syndiniales, which spends the transient dinospore stage as a gamete (Cachon and Cachon 1987), is comparable to that of *Noctiluca*. *Amoebophrya* has typical eukaryotic chromosomes throughout its life cycle (Fensome *et al.* 1993). An SSU rDNA tree showed that *Amoebophrya* species and *N. scintillans* branched off at the base of the typical dinoflagellates (Gunderson *et al.* 1999; Saldarriaga *et al.* 2001). Taylor (1999) explained the basal phylogenetic positions

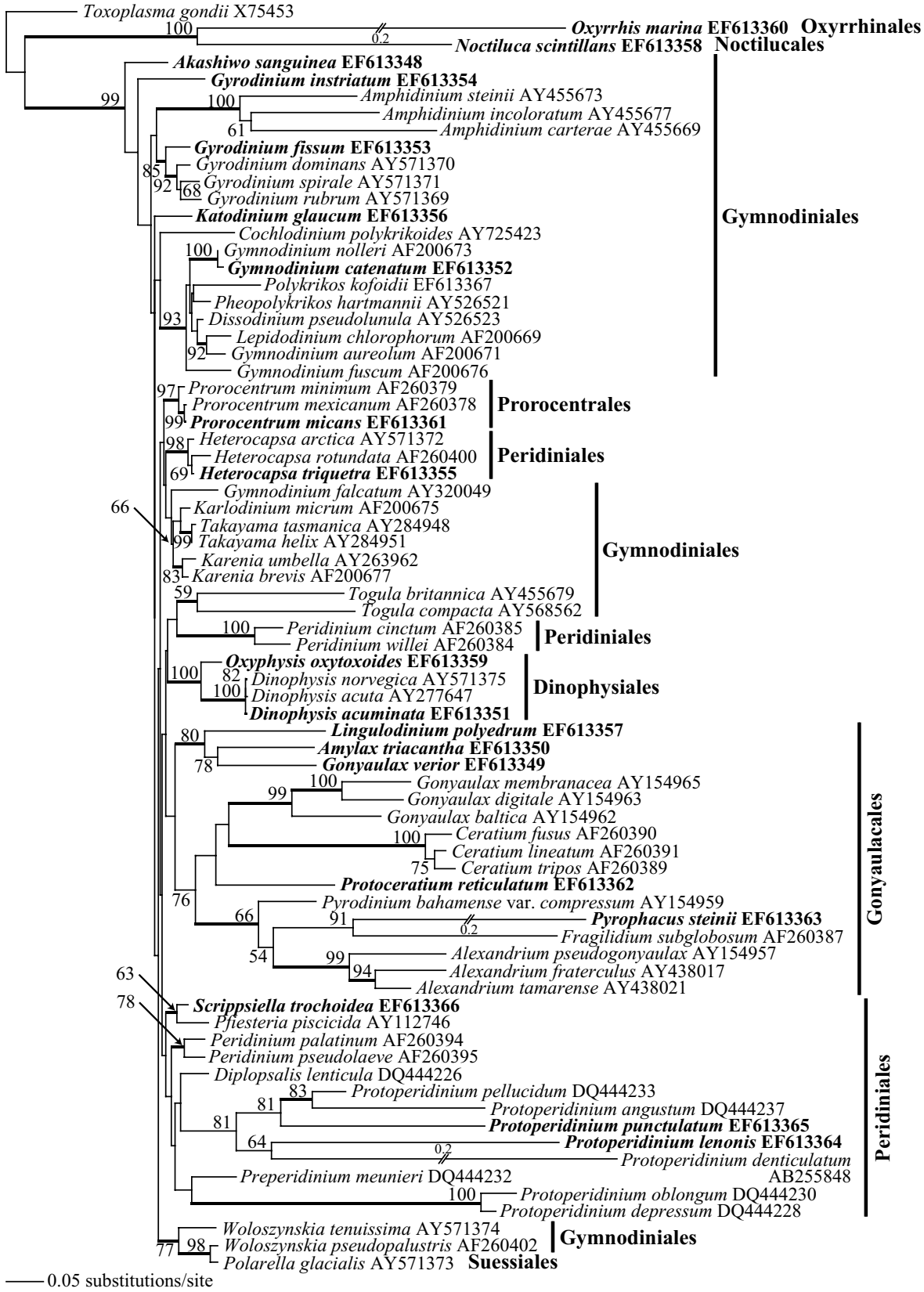

Fig. 1. Neighbor-joining (NJ) tree from the maximum-likelihood distance model reconstructed from nucleotide sequences of dinoflagellate LSU rDNA D1 and D3 domain. NJ bootstrap values ≧ 50% are indicated at branch nodes. Bayesian posterior probabilities ≧ 0.95 are represented by thick lines. Branch lengths are proportional to the number of substitutions per site. The apicomplexan *Toxoplasma gondii* was used to root the tree. The dinoflagellate species in bold were investigated in this study.

of *Amoebophrya* and *Noctiluca* using the gradual histone loss model; the parasitic syndinians with histones always present are the most primitive, followed by noctilucoids and parasitic blastodinians with histones during a part of their life cycle, and typical dinoflagellates are the most derived because they lack essential histones during all stages of their life cycle. This hypothesis seems to be highly congruent with molecular phylogenies constructed using rDNA molecules.

Phylogeny of the Dinophysiales

The Dinophysiales is a distinct dinoflagellate group that is characterized by a unique sagittal suture. This order was the only higher taxonomic-level clade that formed a monophyletic group. The LSU rDNA phylogeny showed that the Dinophysiales, represented by *D. acuminata*, *Dinophysis acuta* Ehrenberg, *Dinophysis norvegica* Claparède et Lachmann, and *O. oxytoxoides*, formed a monophyletic assemblage with 100% BS and 1.00 PP. *Oxyphysis oxytoxoides* was placed at the most ancestral position relative to the monophyletic *Dinophysis* Ehrenberg species.

Edvardsen *et al.* (2003) found that *Dinophysis* species formed a monophyletic group in which heterotrophic *Dinophysis rotundata* Clarepède et Lachmann diverged first. This heterotrophic *Dinophysis* species appears to be a phylogenetic intermediate between heterotrophic *O. oxytoxoides* and autotrophic *Dinophysis* species. The genus *Oxyphysis* Kofoid is considered to be a link between the Dinophysiales and the Peridiniales because of its morphological similarities to *Oxytoxum* Stein (Steidinger and Tangen 1996). However, there was no apparent phylogenetic relationship between the *Dinophysis/Oxyphysis* clade and any peridinialean species.

Phylogeny of the Gonyaulacales

The NJ and BI trees inferred from LSU rDNA sequence data showed that members of the Gonyaulacales consistently grouped together, but their monophyletic nature was not supported statistically (Fig. 1). They were further subdivided into three taxonomic groups: the families Ceratiaceae, Goniodomataceae, and Gonyaulacaceae. The former two families formed monophyletic groups, but the latter was polyphyletic. There were no interfamilial relationships among them. This phylogenetic relationship is congruent with a previous one based on SSU rDNA data (Saldarriaga *et al.* 2004).

All members of the Goniodomineae, i.e., *Alexandrium* Halim species, *Fragilidium subglobosum* (von Stoch) Loeblich III, *Pyrodinium bahamense* Plate var. *compressum* (Böhm) Steidinger, Tester et Taylor, and *Pyrophacus stenii*, formed a monophyletic group with 66% BS and 0.99 PP. The monophyly of the Goniodomineae species is congruent with their homologous morphological characteristics such as the absence of the Q platelet, the hypothecal organization in the quinqueform 2"", and the comma-shaped or slit-like apical pore complex (APC; Dodge and Hermes 1981; Fensome *et al.* 1993). Of these species, *P. bahamense* var. *compressum*, which is the most distinct due to a tabulation pattern in which the posterior sulcal platelet (Sp) and the Ssa are located inside the sulcus (Fensome *et al.* 1993), appears to be an evolutionary intermediate between the Goniodomineae with a quinqueform 2"" and the Gonyaulacineae and Ceratineae with a sexiform 2"".

Pyrophacus steinii showed the closest genetic affiliation to *F. subglobosum* in the LSU rDNA tree, with 91% BS in the NJ tree. Fensome *et al.* (1993) included the somewhat atypical, extremely multiplated *Pyrophacus* Stein in the Goniodomineae based on a number of homologous features such as the cyst shape, the first apical plate (1'), the APC, and the antapical plate patterns. In addition the occurrence of a small thecate gamete with a reduced number of thecal plates has been reported during a part of the life cycle of *Pyrophacus* species (Montresor and Marino 1994; Pholpunthin *et al.* 1999). Thus, the familial assignment of *P. steinii* is well supported by morphological and molecular data. This species formed the longest branch in the rDNA tree, which seems to correspond to its atypical, derived morphology and pleomorphic life cycle.

Three *Ceratium* species, representing the Ceratiaceae, were recovered as a monophyletic group with 100% BS and 1.00 PP. This phylogenetic relationship is justified by the combination of the absence of the Q platelet, the presence of an additional postcingular plate, the sexiform 2"", the tubular APC, and the prominent horns (Dodge and Hermes 1981; Fensome *et al.* 1993).

The LSU rDNA tree revealed that the Gonyaulacaceae, which are distinguished by the combination of the Q platelet, a sexiform 2"", and a tubular APC (Dodge and Hermes 1981; Fensome *et al.* 1993), was recovered as a polyphyletic group. *Amylax triacantha* and *G. verior* showed the closest genetic affiliation and formed a strongly supported monophyletic group with *L. polyedrum* (80% BS and 1.00 PP). These three dinoflagellate species branched first among members of the

Gonyaulacales, without a confident phylogenetic affiliation. All other gonyaulacalean species formed a monophyletic group with 76% BS in the NJ tree. Within this group, *Gonyaulax* Diesing species formed a strongly supported monophyletic group, whereas *P. reticulatum* showed no clear phylogenetic relationship.

Amylax triacantha and *L. polyedrum* have an identical thecal formula of APC, Q, 5', 6", 5'", 2"", which justifies their classification outside the genus *Gonyaulax*, which has a thecal formula of APC, Q, 4', 6", 5'", 2"" (Sournia 1984; Dodge 1989). The two species are distinguishable by the position of the Q platelet against the APC (Dodge 1989). Further, *G. verior* has a thecal formula homologous to that of typical *Gonyaulax* species. However, this dinoflagellate species shares the strong antapical spine(s) and dorsoventral compression with *A. triacantha* and produces a smooth and oval cyst (Mastuoka *et al.* 1988) that is clearly different from that of the *Gonyaulax* group, which produces cysts similar to those of the cyst-based genera *Bitectatodinium* and *Spiniferites* (Ellegaard *et al.* 2003). These morphological similarities appear to support the close phylogenetic affiliation between *G. verior* and *A. triacantha*. The SSU rDNA tree of Saldarriaga *et al.* (2004) also showed consistent clustering of *G. verior* (as *A. diacantha*) and *L. polyedrum*. Re-observation of the thecal formula of *G. verior* or redefinition of the genus *Amylax* is needed in the future study.

The NJ tree did not show a phylogenetic relationship of *P. reticulatum* with any other gonyaulacalean species, whereas the BI tree showed a more or less close relationship between this dinoflagellate species and *Gonyaulax* species. Of the gonyaulacacean members, *P. reticulatum* is atypical in that it has a narrow slit-like APC, does not have the Q platelet (Reinecke 1967; von Stosch 1969; Hansen *et al.* 1996/97), and has a spherical cyst with numerous processes bearing capitate distal ends (Mastuoka and Fukuyo 2000). This dinoflagellate species clearly differs from typical *Gonyaulax* species in the thecal formula and cyst shape, but Hansen *et al.* (1996, 1996/97) identified a close relationship between *P. reticulatum* and *Gonyaulax* species on the basis of homologous ultrastructural features.

Phylogeny of the Gymnodiniales

In the LSU rDNA tree, there were notable phylogenetic affiliations of *Dissodinium pseudolunula* Swift ex Elbrächter et Drebes, *Pheopolykrikos hartmannii* (Zimmerman) Matsuoka et Fukuyo, and *Polykrikos kofoidii* Chatton to *Gymnodinium* (Stein) G. Hansen et Moestrup species, with 93% BS and 1.00 PP. There was also a more or less close phylogenetic affiliation among *Karlodinium* J. Larsen, *Karenia* G. Hansen et Moestrup, and *Takayama* de Salas, Bolch, Botes et Hallegraeff, with 66% BS in the NJ tree. There were no genetic affinities among athecate dinoflagellate species or genera such as *Akashiwo* G. Hansen et Moestrup, *Amphidinium* (Claparède and Lachmann) Flø Jørgensen, Murray et Daugbjerg, *Cochlodinium* Schütt, *Dissodinium* (Klebs ex Pascher) Elbrächter et Drebes/*Gymnodinium*/*Pheopolykrikos* (Chatton) Matsuoka et Fukuyo/*Polykrikos* Bütschli, *Gyrodinium* (Kofoid et Swezy) G. Hansen et Moestrup, *Gyrodinium falcatum* Kofoid et Swezy (as *Gymnodinium falcatum*), *Karenia*/*Karlodinium*/*Takayama*, *Katodinium* Fott, and *Togula* Flø Jørgensen, Murray et Daugbjerg. Athecate dinoflagellates are a heterogeneous assemblage (Fensome *et al.* 1993; Daugbjerg *et al.* 2000; Saldarriaga *et al.* 2001, 2004). Our molecular phylogeny clearly implies that the absence of thecal plates is not taxonomically informative and should not be used for taxonomic assessment at higher taxonomic levels (i.e., the Gymnodiniales).

The genera *Gymnodinium* sensu lato and *Gyrodinium* sensu lato were conventionally distinguished by a cingulum displacement (Kofoid and Swezy 1921). However, Daugbjerg *et al.* (2000) suggested that the two genera were unnatural taxonomic groups based on morphological, physiological, and molecular characteristics. Subsequently, Daugbjerg *et al.* (2000) reappraised *Gymnodinium* and moved the gymnodinioid species that did not fit their reappraisal to the newly established genera *Akashiwo*, *Karlodinium*, and *Karenia*. The new genus *Takayama* was also established based on a combination of morphology, pigment composition, and molecular data (de Salas *et al.* 2003). *Karenia*, *Karlodinium*, and *Takayama* are clearly characterized by linear apical grooves and fucoxanthin-containing chloroplasts and form a short-branched, monophyletic group (Daugbjerg *et al.* 2000; de Salas *et al.* 2003). More recently, *Amphidinium* was emended to encompass athecate species with a minute left-deflected epicone (Flø Jørgensen *et al.* 2004a), and *Togula* was newly established to encompass athecate species with a toga-shaped cingulum (Flø Jørgensen *et al.* 2004b).

In our LSU rDNA tree, *D. pseudolunula*, *Gymnodinium* sensu stricto species, *P. hartmannii*, and *P. kofoidii* formed a strongly supported monophyletic group. Their clustering can be explained by morphological features such as the apical groove encircling the apex counterclockwise

and a *Gymnodinium*-like motile stage during a part of their pleomorphic life cycle (see Kim 2005 for more detail).

Heterotrophic *K. glaucum* is a small athecate species that has a postmedian cingulum with a distinct tongue-shaped apical notch (Takayama 1985). It is widely recognized that the genus *Katodinium*, which is characterized by a much larger epicone than hypocone (Dodge 1982), is a polyphyletic group (Hansen 1995; Steidinger and Tangen 1996). For example, Hansen (1995) moved *Katodinium rotundatum* (Lohmann) Loeblich III to the genus *Heterocapsa* Stein. *Heterocapsa* species formed an independent branch that was clearly separated from *K. glaucum* in the phylogenetic tree. The phylogenetic resolution of *K. glaucum* requires sequence analyses of taxonomically related species such as *Katodinium glandulum* (Herdman) Loeblich III, *Katodinium asymmetricum* (Massart) Loeblich III, and *Herdmania litoralis* Dodge, all of which share a distinct apical notch, as well as *Katodinium nieuportense* (Conrad) Loeblich Jr. and Loeblich III, which is the type species (Dodge 1982).

According to Daugbjerg *et al.* (2000), *Gyrodinium* sensu stricto is characterized by its heterotrophic nutritional mode and elliptical apical groove making a complete circuit. Our molecular data showed that *Gyrodinium* species formed a monophyletic branch with 85% BS and 0.98% PP. Of these species, *G. fissum* was located at the most basal position and gave rise to a monophyletic clade composed of *Gyrodinium dominans* Hulburt, *Gyrodinium rubrum* (Kofoid et Swezy) Takano et Horiguchi, and *Gyrodinium spirale* (Bergh) Kofoid et Swezy. The taxonomic reappraisal of *Gyrodinium* was supported by Hansen and Daugbjerg (2004) and Takano and Horiguchi (2004), based on molecular and ultrastructural data.

Our phylogenetic tree shows that photosynthetic *G. instriatum* showed no phylogenetic relationship to typical *Gyrodinium* species or other athecate dinoflagellate genera. In an SSU rDNA phylogeny, *G. instriatum* formed an apparently monophyletic cluster with *Gyrodinium dorsum* Kofoid et Swezy and *Gyrodinium uncatenum* Hulburt (Kim *et al.* 2004b; Saldarriaga *et al.* 2004). The distinct phylogenetic position of *G. instriatum* among the dinoflagellate lineages can be explained by the characteristic shape of the apical groove encircling the apex once, with the distal and proximal ends reaching the upper junction of the cingulum and the sulcus (Takayama 1985), and by peridinin as the major carotenoid (unpublished data). Given the great differences in cell morphology, pigment composition, and molecular data, the separation and establishment of a new genus for the three photosynthetic *Gyrodinium* species is inevitable.

Phylogeny of the Peridiniales

The Peridiniales is defined taxonomically by bilaterally symmetrical tabulation and the APC with a canal platelet, X (Fensome *et al.* 1993). In the LSU rDNA tree, species belonging to the same genus formed independent or well-supported monophyletic clusters, i.e., those of *Diplopsalis* Bergh, *Heterocapsa*, *Preperidinium* Mangin, and *Scrippsiella* Balech ex Loeblich III, except for those of *Peridinium* Ehrenberg and *Protoperidinium* Bergh. *Protoperidinium* species form distinct morphological groups based on the shape of the 1' and the secondary anterior intercalary plate (2a; Gribble and Anderson 2006; Yamaguchi *et al.* 2006). As previously reported, *Protoperidinium conicum* (Gran) Balech, *P. leonis*, *Protoperidinium pentagonum* (Gran) Balech, and *P. punctulatum*, belonging to the section Conica with an ortho 1' and a hexa 2a, formed a polyphyletic group (Gribble and Anderson 2006; Yamaguchi *et al.* 2006). Interestingly, *Scrippsiella* species were related to *Pfiesteria piscicida* Steidinger et Burkholder with more or less strong statistical support (63% BS and 1.00% PP), a relationship that is not congruent with the SSU rDNA phylogeny (Saldarriaga *et al.* 2001, 2004). Other than this relationship, no other clusters had reliable genetic affinities to each other or to other dinoflagellate species.

Phylogeny of the Prorocentrales

Previous studies found that the genus *Prorocentrum* Ehrenberg, belonging to the Prorocentrales, was a polyphyletic group with species sorting into two main clades in the SSU rDNA tree (Grzebyk *et al.* 1998; Oldach *et al.* 2000). Limited LSU rDNA data were available for *Prorocentrum* species, so its polyphyletic nature was not examined further.

CONCLUSION

We presented the LSU rDNA sequences of 19 dinoflagellate species occurring in Korean coastal waters. The phylogenetic tree inferred from the partial LSU rDNA sequences resulted in the following notable findings. First, *Noctiluca scintillans* and *O. marina* branched at the most basal positions of the dinoflagellate lineage, congruent with their atypical morphological and cytological features. Second, the Dinophysiales, represented by

Dinophysis species and *O. oxytoxoides*, formed a strongly supported monophyletic assemblage. Third, *Gyrodinium instriatum* formed an independent branch separated from typical *Gyrodinium* species, consistent with its distinct apical groove and autotrophism. Fourth, *Katodinium glaucum*, which has a distinct apical notch, also formed an independent branch with no close phylogenetic affiliations to any other dinoflagellate species. Fifth, members of the Gonyaulacales were subdivided into distinct taxonomic groups whose phylogenetic relationships were generally congruent with differences in the combinations of APC shape, hypothecal organization, and thecal formula.

Sequence data from rDNA molecules are useful not only for resolving the phylogenetic relationships and evolutionary histories of the dinoflagellate lineages, but also for identifying dinoflagellate species with taxonomic ambiguity. However, rDNA molecules suffer from high mutational saturation, and the phylogenetic relationships among the dinoflagellate lineages were fundamentally irresolvable. To resolve fine-scale phylogenetic relationships, future studies should not only include novel taxa, but should also investigate alternative molecular markers (e.g., Saldarriaga *et al.* 2003; Shalchian-Tabrizi *et al.* 2006).

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